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Post-seizure drug treatment in young rats determines clear incremental losses of frontal cortical and hippocampal neurons: The resultant damage is similar to very old brains

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ABSTRACT

Loss of neurons occurs with aging and following lithium/pilocarpine-induced epileptic seizures. In the present study, the numbers of neurons within the layers from sample areas of the four lobes of the neocortices and the hippocampus were counted by light microscopy in brains of rats that had been administered lithium or pilocarpine and then injected immediately or shortly after seizure onset with either acepromazine, ketamine, or prazosin. The mean numbers of neocortical and hippocampal neurons were lowest in rats treated with acepromazine or prazosin 1 h after seizure onset, while those of rats immediately treated with ketamine displayed the least decrements and were most similar to normal rats. The largest loss of neurons occurred within the CA1 field and layers 5 and 6 of the frontal cortices. The mean numbers of neurons within the cortices in rats whose treatments had been delayed for 1 h were similar to those of normal rats over 700 days of age. These results support the hypothesis that neuronal loss from cumulative effects of seizure-induced brain damage simulates aging.

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1. Introduction

A sustained epileptic seizure, *status epilepticus*, is associated with significant loss of neurons and accentuated risk of mortality. This is evident in the display of overt rearing, rapid forelimb clonus and electroencephalographic patterns of paroxysmal activity [1] within about 30 min in rats after a single subcutaneous injection of lithium (3 mEq/kg) and 4 to 24 h after a 30-mg/kg injection of pilocarpine. There is significant mortality unless post-seizure pharmacological treatments are administered [1,2].

The injections of different classes of post-seizure drugs that promote survival are associated with markedly different outcomes for subsequent behaviors. For example, seized rats injected with ketamine rather than acepromazine respond similarly to non-seized controls in various spatial memory tasks [3–5]. Even the onset of delayed extreme obesity, following the induction of these seizures in prepubescent female rats, is determined by a single post-seizure injection of acepromazine but not of ketamine [6].

In our original approach to this problem, we appreciated an opportunity to study the synergistic interactions between different receptor subtypes on different populations of neurons and their pharmacological ligands as a potential model to study differential patterns of epilepsy-induced brain damage and how treatments might be developed to ameliorate these patterns as well as the anomalous behaviors that followed. Even those seized rats that received compounds such as ketamine and displayed no conspicuously abnormal behaviors were vulnerable to influence from other stimuli to which the normal rats or the seized rats receiving other compounds were oblivious [7].

One of the most conspicuous observations noted in the learning and memory of seized rats administered acepromazine was their marked similarity to very old rats. Consequently, we decided to compare the brains of rats that had received various single post-seizure treatments to our population of aged rats to discern if this intrinsic hypothesis was supported. If the numerical values for seizure-induced neuronal loss in young brains are comparable to those associated with very old (geriatric) brains, then different approaches to both pharmacological treatment and strategies for adaptation might be considered.

There have been many qualitative and semi-quantitative studies regarding the regions of the rat brain that are damaged as revealed by necrosis, gliosis, and the formation of cystic lesions over time [8–12]. The most devastated areas involve the piriform and entorhinal cortices, the hippocampus, the substantia nigra reticulata, specific amygdaloid nuclei, and a variety of thalamic nuclei such as the reuniens, lateral posterior groups, and subnuclei of the medial geniculate and dorsomedial thalamus [13]. The CA1 field of the hippocampus is obviously

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decimated. However, the changes within the cerebral cortices are often considered, qualitatively at least, minor.

In the present study, we focused upon the layers of various regions of the cerebral cortices and the hippocampus. Unlike previous approaches, direct quantitative measurement of the numbers of neurons rather than nominal estimates or rank order of missing neurons was utilized. We report here that a single injection of different drugs immediately or 1 h after overt indicators of epileptic seizures produced conspicuous quantitative decrements in the density of neurons. The more severe changes are comparable to neuronal densities observed in old (>700 days) rats.

2. Method

2.1. Subjects

Brains (n = 35) from rats that had been employed in a previously unpublished experiment were obtained from our library. Brains (n = 5) from age-matched female normal rats were also obtained. The cerebrums of all the brains (fixed in ethanol–formalin–acetic acid solution and embedded in paraffin) had been sectioned by a microtome such that 50- and 10-μm sections between the posterior commissure and the anterior commissure had been obtained. There were 7 brains in each of the 5 conditions that were available from our unpublished study. At around 90 days of age, all of these female rats had been injected subcutaneously with 3 mEq/kg of lithium in the left flank and then, 4 h later, with 30 mg/kg of pilocarpine in the right flank.

After the onset of a type IV seizure (rearing and rapid forelimb clonus), the rats were injected intraperitoneally with either acepromazine (30 mg/kg) or ketamine (100 mg/kg) immediately or after 1 h with one of the two drugs. A fifth group was injected with prazosin (5 mg/kg) after 1 h. (The brains of the group injected immediately with prazosin were not available.) The rats had been killed by decapitation about 50 days later (150 days of age). The brains were quickly fixed in ethanol–formalin–acetic acid solution.

2.2. Procedure

Sections from the same level of the occipital, temporal, and parietal lobes and sections from two different regions of the frontal lobes for each rat were stained with toluidine blue O. The two frontal regions were considered specific motor (Fp1) compared with an analog of the prefrontal (Fp2) cortices found in human beings. For each brain, for each area, and for each layer of the cortices, the numbers of neurons were counted at 400× in four different areas of the same layer. The mean value was used as the measure for that layer. A 6×6-grid system was employed to insure accuracy. The area within the 6×6 grid at 400× was 0.09 mm^2. Because there is no discernable layer 4 in the prefrontal cortices, the numbers of neurons in the lower portion of layer 3 and the upper portion of layer 5 were averaged to allow symmetry in the analyses. For the hippocampus, the numbers of neurons within the CA1, CA2 and CA3 fields were also enumerated in a similar manner.

2.3. Statistics analyses

The basic design for the cerebral cortices was a three-way analysis of variance with one between (5 post-seizure drug treatments and normal reference brains) and two within (layer and lobe) factors. For the hippocampus, the design was a two-way analysis of variance (treatment vs CA field). Post hoc analysis involved various combinations of t-tests for both main effects and interactions. Factor analyses and other ancillary procedures were also explored to isolate sources of variance. All analyses involved SPSS PC 16.

2.4. Comparison to age-span brains

To test the hypothesis of the aging simulation from epileptic neuronal damage, the curves for the mean neurons within the cerebral cortices and the hippocampus were obtained from our database. The age-span database [14] involved 32 brains from rats that had been between 30 and 940 days of age at the time of death.

3. Results

3.1. Post-seizure drug treatment effects

The most salient relationship associated with the numbers of neurons still viable about 50 days after lithium/pilocarpine-induced seizures and the different post-seizure treatments is reflected by the scattergram in Fig. 1. It shows the correlation between the mean numbers of cells per unit area for all of the cerebral cortical areas measured and the numbers of cells in the CA fields of the hippocampus. The different treatments are indicated within the figure. All seized rats, regardless of treatment, displayed fewer neurons than their age-matched controls. Ketamine treatment immediately after seizure onset resulted in the least loss of neurons while prazosin treatment 1 h after seizure onset produced the most loss. The discontinuity of groups (below the horizontal line) illustrates the effect.

The more formal analyses of variance for the three CA fields as a function of treatment (5 drugs and control) demonstrated a statistically significant [$F(5,34) = 321.93$, $p < .001$; $eta^2 = 0.98$] difference between groups for the average numbers of neurons in all fields. Post hoc analyses indicated that all groups were significantly different from each other. The statistically significant interaction [$F(10,68) = 36.96$, $p < .001$; partial $eta^2 = .84$] between treatment and CA field was due primarily to the greater loss of neurons within the CA1 field for the prazosin treatment (M = 54, SD = 6) compared with the controls (M = 109, SD = 4), whereas this difference was not as evident in the CA2 (M = 50, SD = 3; M = 76, SD = 1) and CA3 (M = 56, SD = 3; M = 74, SD = 2, respectively) fields. Other smaller group differences contributed in a smaller manner but displayed the same pattern. Compared with the CA1 field that showed a significant (Tukey's $p < .05$) difference between each group, there were no significant differences in the numbers of neurons for the 1-h-delayed acepromazine- or prazosin-treated brains. For the CA3 field, there was no significant difference between the 1-h-delayed acepromazine- and prazosin-treated

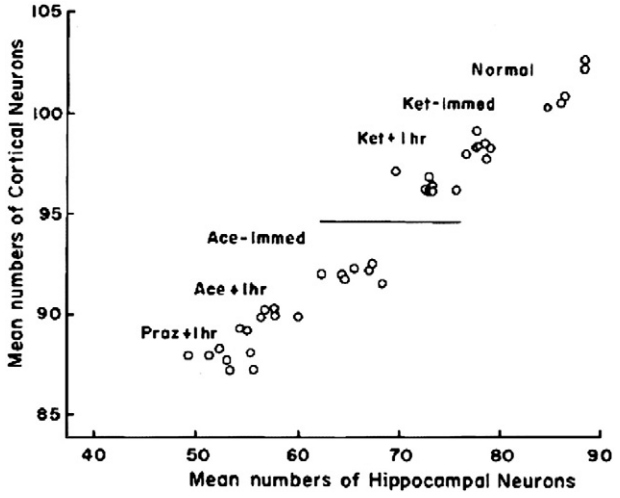

Fig. 1. Mean numbers of neurons per 0.09 mm^2 (400×) for all areas and layers within the cerebral cortices, and mean numbers within the CA1 to CA3 fields of the hippocampus as a function of post-seizure drug treatment. The means of all groups were significantly different from each other.

Table 1
Means and standard deviations for the numbers of neurons per unit area for different cortical layers averaged from all six locations. The difference between any pair of means can be considered significant statistically if it exceeds the mean standard deviation of the pair.

Treatment	Layer 1		Layer 2		Layer 3		Layer 4		Layer 5		Layer 6	
	M	SD	M	SD	M	SD	M	SD	M	SD	M	SD
Control	14	2	70	3	146	2	108	1	133	1	140	3
Ket. immed.	13	1	73	1	146	1	107	2	128	1	129	2
Ace. immed.	9	1	72	1	141	2	103	1	117	1	116	1
Ket. +1 h	12	1	74	1	146	2	106	1	125	1	123	1
Ace. +1 h	8	1	71	1	138	2	100	1	114	1	111	1
Praz. +1 h	7	1	68	1	135	2	98	1	111	1	107	1

brains or between the normal and immediately treated ketamine brains.

A three-way analysis of variance for the mean numbers of neurons in all layers of the cerebral cortices for all five locations demonstrated statistically significant [$F(5,34) = 628.195$, $p < .001$; $eta^2 = .99$] group differences. Post hoc analysis indicated that each group's mean numbers of neurons were different from those of each of the other groups. The significant interaction between layer and condition [$F(25,170) = 70.44$, $p < .001$] was shown by post hoc analyses to be primarily due to the greater loss of neurons within layers 5 and 6 for all brain areas for the 1-h-delayed acepromazine- and prazosin-treated rats compared with other treatments or the controls (Table 1). Within the layers of the cortices, the differences between the six treatments were all statistically significant [$F(5,34) = 693.38 < 15.80$, $p < .001$].

The means and standard deviations for the numbers of neurons for the five locations are shown in Table 2. Post hoc analyses verified that the major effect of the interaction was the greater loss of neurons within the frontal and temporal cortices as a function of the different post-seizure drugs where there was negligible loss of neurons following any of the treatments, compared to age-matched normal controls, within the parietal and occipital regions. The statistically significant interaction between cortical layers, lobe, and treatment [$F(100,680) = 15.25$, $p < .001$; partial $eta^2 = .69$] was due primarily to the greater loss of neurons in layers 5 and 6 of the 1-h-delayed acepromazine- and prazosin-treated rats compared with other treatments with minimal treatment differences for the upper cortical layers.

3.2. Comparisons with aged brains

Fig. 2 shows the mean numbers of neurons for all layers sampled from each lobe from our pool of rat brains between the ages of 30 and 940 days. The immediately treated or 1-h-delayed ketamine-treated brains of those approximately 140-day-old rats were similar to the 400- to 600-day-old normal brains. The brains of the immediately treated and 1-h-delayed-treated rats were more similar to the brains of very old rats. What was particularly significant was the marked aging equivalence of neuronal density in the cerebral cortices of rats that were seized and treated with acepromazine or prazosin.

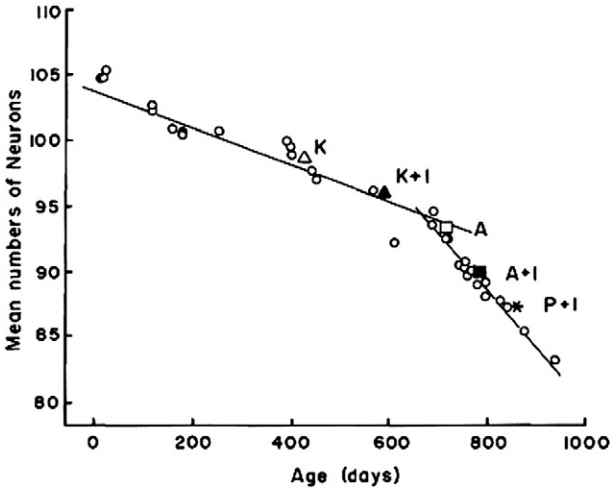

Fig. 2. Mean numbers of neurons as a function of age. The mean numbers of neurons per 0.09 mm^2 for rats that received various post-overt seizure treatments are also shown. K = immediate ketamine, K + 1 is ketamine 1 h after seizure onset, A is immediate acepromazine, A + 1 is acepromazine 1 h after seizure onset and P + 1 is prazosin 1 h after onset. The means of all groups were significantly different from each other.

This resulted in neuronal densities typical of very old rats that had entered the rapid decline often associated with "geriatric" conditions.

4. Discussion

Protracted seizures, *status epilepticus*, lead to significant neuronal damage and death. The type of post-seizure drug treatment significantly affects the ultimate number of neurons remaining within the cerebral cortices and the hippocampus. The vulnerability of the hippocampus, particularly the CA1 sector of the hippocampus, Sommer's sector, to hypoxia and epileptic injury has been known for decades [14]. Lithium/pilocarpine-induced seizures produce a significant loss of neurons within this region.

Most of our measurements of neuronal loss in past publications have been based upon references to control brains by ranking scores or by the visually conspicuous presence or absence of neurons and the presence of gliosis or cystic lesions within the affected area. The results of the present study emphasize the importance of laborious quantitative measurements of neurons. In the past, visual inspections of the cerebral cortices by a variety of experienced light microscopists did not reveal the presence of neuronal diminishment within the neocortices (particularly the frontal area) in rats in which seizures have been induced. The qualitative effects that were evident were minute in comparison with the ranks of devastation within the subcortical telencephalic and diencephalic structures [13].

The results presented here indicate that the numbers of neurons within the cortices that were lost following seizure inductions were directly determined by the type of drug and the latency of injection. Whereas neurons within layer 2 did not appear vulnerable to the

Table 2
Means and standard deviations for the numbers of neurons per unit area for the different locations (mean of all layers). The difference between any pair of means can be considered significant statistically if it exceeds the mean standard deviation of the pair.

Treatment	Frontal 1		Frontal 2		Temporal		Parietal		Occipital	
	M	SD	M	SD	M	SD	M	SD	M	SD
Control	107	2	102	4	91	2	102	2	108	1
Ket. immed.	105	1	95	1	86	1	103	1	108	1
Ace. immed.	98	1	80	1	77	1	101	2	108	1
Ket. +1 h	102	2	91	1	82	1	103	1	109	2
Ace. +1 h	93	1	76	1	75	1	101	1	107	1
Praz. +1 h	92	2	69	1	73	1	99	1	106	1

seizures or treatments, the neurons in layers 5 and 6 exhibited significant neuronal dropout. Ketamine injected immediately and even 1 h after seizure onset minimized neuronal loss. Several behavioral studies have shown that seized rats injected with ketamine behave remarkably similar to non-seized controls [3–5]. Other NMDA receptor antagonists such as CGP 40116 also produce qualitatively conspicuous reduction in neuronal damage [15].

The differences in neuronal loss were greatest within layers 5 and 6 of the frontal cerebral cortices. These neurons are major inputs to the caudate–putamen whose output through the globus pallidus to the thalamic sources contributes to the loop that helps maintain cerebral cortical–basal gangliar–thalamic activity. A particular dysfunction in this pathway is also suggested by the forms of the spontaneous seizures that arise in these populations after a latency of a few weeks. The pattern involves rearing and rapid forelimb clonus, a pattern of stereotyped motor activity wherein neurons residing within the portion of the frontal cortices were measured.

The remarkable absence of difference in neurons within normal and seized cortices, regardless of post-seizure treatment, within the occipital and parietal lobes indicates that the processes leading to cell loss were not diffuse. Hence, the dropout within the lower layers of the frontal lobe was not secondary to some generalized metabolic or vascular limitation unless the prefrontal neurons are more sensitive to hypoxia or metabolic transients to which other cortices do not respond. The specific loss of frontal neurons would be expected to affect planning and inhibitory behavior which has been shown in several previous experiments [16,17]. Mineralocorticoid receptors that display an affinity for corticosteroids about 10 times higher than glucocorticoid receptors show preferential distribution in the prefrontal cortices [18].

Direct comparison of the numbers of neurons from seizure induction to the numbers of neurons within aged animals confirms our clinical hypothesis on cerebral changes following closed-head injuries, and resultant increase in complex partial epileptic-like conditions in human beings [19] simulates the effects of “normal” aging. However the changes occur within weeks rather than decades. Except for the ketamine-treated seized rats, the numbers of neurons within the cerebral cortices of rats treated with other post-seizure compounds, in order to maintain the viability of the organism, were more typical of normal rats over the age of 700 days. In fact, the more severe neuronal losses, such as those treated with prazosin delayed by 1 h, overlap with the rapidly declining stage of aging. The behaviors of very old normal rats have rarely been explored. However, in our many unpublished experiments over the years, geriatric rats (>2 years of age) also perform poorly in maze conditions and engage in repetitive behaviors.

The discontinuity in slope around 700 days of age and the subsequent accelerated loss of neurons (Fig. 2) in very old normal rats might be considered comparable to the accelerated loss in brain mass within the human brain after 80 years of age [20]. Whereas the mass loss declines slowly from 26 to 80 years of age in human brains, on average about 2 g per year, subsequent decreases involve 5 g per year. For comparative purposes, it is relevant that the 10% loss of neurons between early and late adulthood noted in the rat brain in our study is very similar to the value reported for all neocortical neurons over the life span of both sexes for human beings [21].

The rapid loss of neurons following seizure induction or closed-head injuries that result in the effective equivalence of a geriatric cerebrum has clinical implications. One may speculate that most human beings have time to slowly adapt to the gradual losses of neurons over decades, whereas individuals who have sustained a closed-head injury or displayed epileptic seizures similar to those induced in the present study are suddenly confronted with the same premorbid demands but with the neuronal capacity of a geriatric person. The “slowness” and reduced problem-solving capacity of younger people who sustain such injuries could be viewed as characteristic of the normal older person. Responses such as anxiety, depression, and “psychological difficulties” would be expected in association.

References

[1] Persinger MA, Stewart LS, Richards PM, Harrigan T, O'Connor RP, Bureau YRJ. Seizure onset times for rats receiving systemic lithium and pilocarpine: sources of variability. Pharmacol Biochem Behav 2002;71:7–17.

[2] Persinger MA, Bureau YRJ, Kostakos M, Peredery O, Falter H. Behaviors of rats with insidious, multifocal brain damage induced by seizures following single peripheral injections of lithium and pilocarpine. Physiol Behav 1993;53:849–66.

[3] Santi SA, Cook LL, Persinger MA, O'Connor RP. Normal spatial memory following postseizure treatment with ketamine: selective damage attenuates memory deficits in brain-damage rodents. Int J Neurosci 2001;107:63–75.

[4] Stewart LS, Persinger MA. Ketamine prevents impairment when administered immediately after status epilepticus onset. Epilepsy Behav 2001;2:585–91.

[5] Fournier NM, Persinger MA. The neuromatrix and the epileptic brain: behavioral and learning preservation in limbic epileptic rats treated with ketamine but not acepromazine. Epilepsy Behav 2004;5:119–27.

[6] St-Pierre LS, Persinger MA. Extreme obesity in one year old female rats in which seizures were induced before puberty by lithium/pilocarpine followed by a single injection of acepromazine. Epilepsy Behav 2005;7:411–8.

[7] McKay BE, Persinger MA. Weak, physiologically patterned magnetic fields do not affect maze performance in normal rats but disrupt seized rats normalized with ketamine: possible support for a neuromatrix concept? Epilepsy Behav 2006;8:137–44.

[8] Handforth A, Treiman D. Functional mapping of the early stages of status epilepticus: a ^{14}C-2-deoxyglucose study in the lithium–pilocarpine model in rat. Neuroscience 1995;64:1057–73.

[9] Walton NY, Gunawan S, Treiman DM. Brain amino acid concentration changes during status epilepticus induced by lithium and pilocarpine. Exp Neurol 1990;108:61–70.

[10] Henshall DC, Skradski SL, Meller R, et al. Expression and differential processing of caspases 6 and 7 in relation to specific epileptiform EEG patterns following limbic seizures. Neurobiol Dis 2002;10:71–87.

[11] Dube C, Boyet S, Marescaux C, Nehlig A. Progressive metabolic changes underlying the chronic reorganization of brain circuits during the silent phase of the lithium–pilocarpine model of epilepsy in the immature and adult rat. Exp Neurol 2000;162:146–57.

[12] Pereira de Vasconcelos A, Ferrandon A, Nehlig A. Local cerebral blood flow during lithium–pilocarpine seizures in the developing and adult rat: role of coupling between blood flow and metabolism in the genesis of neuronal damage. J Cereb Blood Flow Metab 2002;22:196–205.

[13] Peredery O, Persinger MA, Parker G, Mastrosov L. Temporal changes in neuronal dropout following inductions of lithium/pilocarpine seizures in the rat. Brain Res 2000;881:9–17.

[14] Gloor P. The temporal lobe and limbic system. N.Y.: Oxford University Press; 1977

[15] Fujikawa DG, Daniels AH, Kim JS. The competitive NMDA receptor antagonist CGP 40116 protects against status epilepticus-induced neuronal damage. Epilepsy Res 1994;17:207–19.

[16] Tanji J, Shima K, Mushiake H. Concept-based behavioral planning and the lateral prefrontal cortex. Trends Cogn Sci 2007;11:528–34.

[17] Neubert FX, Klein MC. What is driving inhibition-related activity in the frontal lobe? J Neurosci 2010;30:4830–2.

[18] Lupien SJ, Lepage M. Stress, memory, and the hippocampus: can't live with it, can't live without it. Behav Brain Res 2001;127:137–58.

[19] Lupien SJ, McEwen BS, Gunnar MR, Heim C. Effects of stress throughout the life span of the brain, behaviour and cognition. Nat Rev Neurosci 2009;10:434–45.

[20] Rushton JP, Ankney CD. Whole brain size and general mental ability: a review. Int J Neurosci 2009;119:691–731.

[21] Pakkenberg B, Gundersen HJG. Neocortical neuron number in humans: effects of sex and age. J Comp Neurol 1997;384:312–20.